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Award Number: W81XWH-10-1-0462

TITLE: Role of the Inflammasome in Asbestos-Induced Mesothelioma Formation

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CONTRACTING ORGANIZATION:

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REPORT DATE: October 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 Oct 2012	Annual	15 Sep 2011 - 14 Sep 2012

4. TITLE AND SUBTITLE
Role of the Inflammasome in Asbestos-Induced Mesothelioma Formation

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0462

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The investigations proposed in this idea award grant are aimed at addressing the requirement of asbestos-induced inflammation in the pathogenesis of malignant mesothelioma (MM). Cancer-related inflammation (CRI) within the tumor microenvironment contributes to tumor progression in many human malignancies. MM tumor samples have hallmarks of CRI including macrophage infiltration and inflammatory cytokine production. We intend to evaluate the role of asbestos-induced inflammation in the formation of MM by genetically and pharmacologically inhibiting inflammasome-mediated inflammation in mouse models of MM and determine whether this physiological response is required for tumor development. The overarching goal is to provide the first in vivoevidence of whether inflammation directly contributes to the development of asbestos-induced MM. The proposed investigations may also implicate inflammasome-mediated CRI as a potential target for the prevention or treatment of MM.

15. SUBJECT TERMS
inflammasome, mesothelioma, asbestos, inflammation

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	7	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

The studies proposed in this Idea Award grant address the requirement of asbestos-induced inflammation in the pathogenesis of malignant mesothelioma (MM). Cancer related inflammation (CRI) within the tumor microenvironment contributes to tumor progression in many malignancies. Tumor samples from MM patients have hallmarks of CRI, including macrophage infiltration and inflammatory cytokine production. In this project, we are examining the role of asbestos-induced inflammation as it relates to the development of MM. This is being done by genetically and pharmacologically inhibiting inflammasome-mediated inflammation in mouse models of MM to determine if this physiological response is required for tumor formation. Our main goal is to ascertain whether inflammation directly contributes to the development of asbestos-induced MM and if inflammasome-mediated CRI could act as a target for the prevention or treatment of MM.

BODY (Aim 1)

Experiments with Asc knockout mice

For experiments proposed in Aim 1, 6 to 8-week-old *Asc* null (-/-), heterozygous (+/-) or wild type (WT) animals were injected with asbestos (400 μg in 500 μl PBS) intraperitoneally (i.p.) every 3 weeks for a total of eight injections (3.2 mg total). A total of 105 animals of different genotypes (35 WT, 35 +/-, and 35 -/-) have now completed the eight injections of asbestos. To date, 18 animals (13 *Asc* +/- and 5 *Asc* -/- animals) have been sacrificed because of asbestos-related illness. Eleven of the 18 mice that were euthanized had developed malignant ascites, which was aspirated to relieve discomfort. Although draining the ascitic fluid (4 - 10 ml) prolonged survival for 2-4 weeks, the mice again developed ascites and were euthanized. At this time, the ascitic fluid was consistently hemorrhagic. The first, non-hemorrhagic ascitic fluid was centrifuged, and then one portion of the collected cells was placed in cell culture for future molecular studies, and a smaller portion was used for a cytospin slide preparation. All remaining clear fluid was stored at -80°C for possible future studies of cytokines and other secreted proteins. The cytospin slide was sent to our Histopathology Facility for cytopathological evaluation to assess the presence of malignant and inflammatory cells.

RESULTS

In addition to the 18 mice that were euthanized, the remaining animals that have completed the 8 rounds of asbestos injections are symptom free at this time. Among the 18 animals that were sacrificed, 11 developed ascites, which was clear or yellowish in 9 mice and hemorrhagic in 2 animals. The latter 2 mice were sacrificed immediately, whereas the 9 others were asymptomatic after removal of the ascitic fluid and were returned to the colony for close observation. As noted in last year's annual report, Giemsa staining of cytospin preparations from ascitic fluids revealed the presence of macrophages and other inflammatory cells as well as MM cells. Some of the ascitic fluid was placed in short-term (2-3 passages) cell culture and then cryopreserved in liquid nitrogen for future *in vitro* and molecular studies. A few (2-4) weeks after the ascitic tap, bloating was again obvious, at which point each of these 9 mice was sacrificed. Various tissues from these animals were sent to our Histopathology Facility, where tissue sections were stained

with H&E and IHC staining and interpretation. Among the 18 animals sacrificed to date, all had granulomatous lesions and 15 had MM. The remaining 3 animals had no obvious evidence of MM. The granulomas contained asbestos fibers and chronic inflammatory cells such as lymphocytes, macrophages, and giant cells surrounding the fibers. All tissue blocks (paraffin embedded) from these animals have been catalogued and saved for future studies.

BODY (Aim 2)

Experiments with Nf2;p16Ink4a/p19Arf +/- mice

For the experiments proposed in Aim 2, 8 to 10-week-old *Nf2;p16Ink4a/p19Arf* heterozygous male mice (abbreviated *Nf2;p16/Arf*+/- mice below) were weighed prior to starting treatment with asbestos and the IL-1R antagonist Anakinra (Kinret™). All animals (35 in each arm) were injected 4 times at 21-day intervals with 800 μg of asbestos (total = 3.2 mg) dissolved in 500 μl PBS. Anakinra was dissolved in citrate buffer and injected intraperitoneal (i.p.) at a concentration of 5 mg/kg body weight (dissolved in 100 μl citrate buffer) 6 hours prior to the first asbestos injection and every third day thereafter. After the initial asbestos injection, Anakinra was given every third day at the same concentration. At the same time, animals in the asbestos-exposed control group were injected i.p. with vehicle control (100 μl citrate buffer) every third day. A small gauge needle (21G for asbestos; 26G for Anakinra or citrate buffer) and different injection sites were used so that one area of the mouse did not become overly sensitized. Animals were sacrificed upon signs of disease, and all tissues in both the peritoneal and pleural cavity were preserved in formalin saline and sent for histopathological assessment.

RESULTS

Asbestos-exposed *Nf2;p16/Arf*+/- are being monitored a minimum of twice daily during this experiment. Once the animals start to show signs of disease (ascites, cachexia, shortness of breath, lethargy, difficulty in moving), they are sacrificed. Some animals had intestinal obstruction due to MM development in the mesentery. To date, a total of 42 *Nf2;p16/Arf*+/- animals (21 from the Anakinra group and 21 from the control group) have been sacrificed. More than 85% (36/42) of animals harbored ascites, 25/36 of which was hemorrhagic (12 from control group and 13 from Anakinra group). Upon necropsy, 6 of 21 animals in the control group had one or more large tumor masses diagnosed as peritoneal MM, while only 2 of 21 animals from the Anakinra group had such a tumor mass. The remaining animals from each group had multiple microscopic tumors that were identified as MM upon pathological examination of tissue sections. Most animals with MM also had granulomas. Importantly, Kaplan-Meier curves (Fig. 1) illustrate a significant survival difference between the two treatment arms of this experiment, with control animals having a median survival of 22.6 weeks while Anakinra-treated animals having a median survival of 33.4 weeks following the initial exposure to asbestos. Moreover, MM tumor burden was decreased in asbestos-exposed mice treated with Anakinra compared to those treated with placebo. These data indicate that Anakinra treatment increases the survival of asbestos-exposed mice by approximately 50%, suggesting that inhibiting inflammation-related IL-1R signaling holds promise as a chemopreventive approach in asbestos carcinogenesis. During the coming year, we will continue to follow the remaining asbestos-exposed animals so that we will hopefully have at least 30 evaluable mice in each arm.

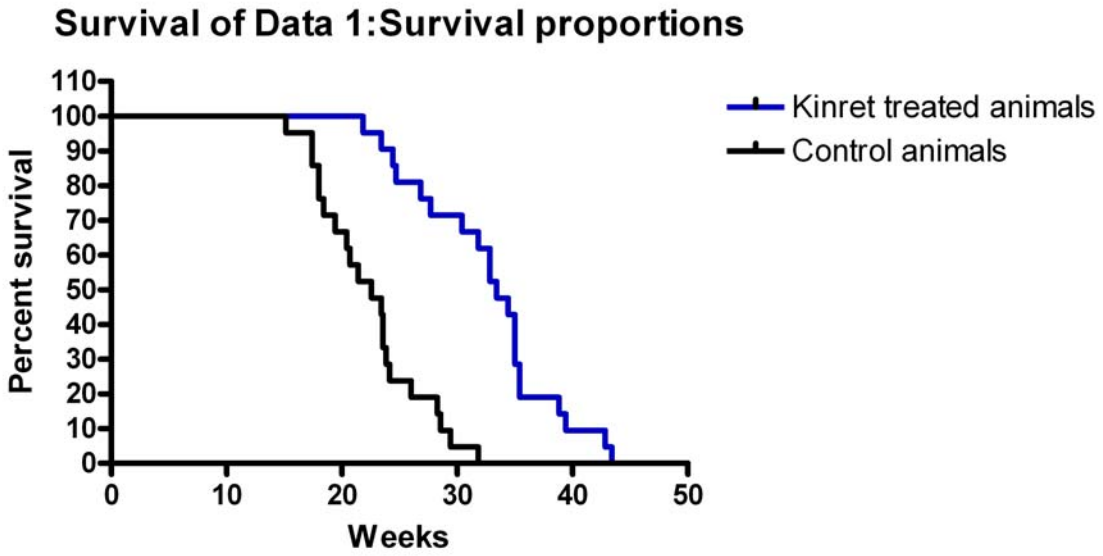

Fig. 1. Kaplan-Meier curves demonstrating that Anakinra (Kinret™) increases the survival of asbestos-induced MM in *Nf2;p16/19* +/- mice given 5 mg/kg body weight of Anakinra (number = 21) or citrate buffer control animals (number = 21) prior to the initial asbestos injection and then every third day thereafter until the animals developed symptoms of MM. Comparison of survival curves is highly significant, with a median survival age of 33.4 weeks for the Anakinra-treated group versus 22.6 weeks for the control animals.

KEY RESEARCH ACCOMPLISHMENTS

- ***Specific Aim 1: Directly determine, using a genetically-defined mouse model, if NALP3-inflammasome-mediated inflammation is required for the induction of MM by asbestos (performed in Years 1 and 2).***

- Task 1. Write and submit animal protocols to do investigations in Specific Aims 1 (months 1-3)
  - 1a) Institutional Biosafety Committee approval to use asbestos **(done)**
  - 1b) IACUC approval for animal protocols **(done)**
  - 1c) DOD approval for animal protocols **(done)**

- Task 2. Perform repeated asbestos injections in male *Asc* +/+, +/- and -/- mice beginning at 6-8 wk of age (months 1-12) **(done)**
  - 2a) Expand animal colony and genotype mice **(done)**
  - 2b) Perform asbestos injections using approved standard operating procedure as male mice are available (35 *Asc* +/-, 35 *Asc* -/- and 35 +/+ males needed) **(done; monitoring of animal health and full histopathological assessment of all tumor tissues continuing)**

• Specific Aim 2: Ascertain if NALP3-inflammasome-mediated IL-1β signaling is required for asbestos-induced MM tumorigenesis, using an IL-1R antagonist.

(performed in years 2 to 3)

- Task 1. Write and submit animal protocols to do investigations in Specific Aims 2 (months 1-3)
  - 1a) Institutional Biosafety Committee approval to use asbestos **(done)**
  - 1b) IACUC approval for animal protocols **(done)**
  - 1c) DOD approval for animal protocols **(done)**
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- Task 2. Perform repeated asbestos injections on male *Nf2;p16/p19* +/- mice beginning at 6-8 wk of age in the presence or absence of a IL-1R antagonist (months 13-24) **(done)**
  - 2a) Expand animal colony and genotype mice **(done)**
  - 2b) Perform asbestos injections using approved standard operating procedure as male mice are available (35 *Nf2;p16/p19* +/- males treated with IL-1R antagonist Anakinra and 35 *Nf2;p16/p19* +/- untreated males) **(asbestos injections done; Anakinra chemoprevention treatment continuing. Also, monitoring of animal health and full histopathological assessment of all tumor tissues continuing)**

REPORTABLE OUTCOMES

All asbestos injections for Aims 1 and 2 completed. Yuwaraj Kadariya, M.D. is successfully overseeing all of the asbestos and Anakinra injections and sampling of tissues. In addition, he is using his medical expertise to carefully identify the most appropriate tissue samples for histopathological assessment.

CONCLUSIONS

We submitted animal protocols that were approved by Fox Chase Cancer Center's IACUC and ACURA to conduct the experiments proposed in this grant proposal, including an addendum that permitted us to drain ascitic fluid to alleviate animal discomfort. In Aim 1, all 35 *Asc* +/+, 35 heterozygous (+/-), and 35 homozygous (-/-) *Asc* mice have completed the asbestos protocol. We have sacrificed 13 *Asc* +/- and 5 *Asc* -/- animals upon development of disease symptoms. To date, no WT (*Asc* +/+) mice have developed MM. Analysis of the WT (*Asc* +/+) mice is ongoing at this time. The Asc gene encodes a component of the NALP3 inflammasome, and the lower incidence of MMs seen to date in *Asc*-null mice than in *Asc* +/- mice suggests that loss of both copies of the *Asc* gene results in decreased or decelerated inflammasome-related MM onset. In Aim 2, data obtained thus far from 42 asbestos-exposed *Nf2;p16/19* +/- mice (21 animals in each arm of treatment) suggest that Anakinra increases survival by about 50% and decreases tumor burden in asbestos exposed mice. If these data hold up upon completion of the studies, the data would demonstrate for the first time that blocking inflammasome-mediated IL-1β processing and release during asbestos exposure might be an efficacious chemopreventive therapy for MM.